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TITLE: Breast Cancer Stimulation of Osteolysis

PRINCIPAL INVESTIGATOR: Merry J. Oursler, Ph.D.

CONTRACTING ORGANIZATION: University of Minnesota
Duluth, Minnesota 55812

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13. ABSTRACT <i>(Maximum 200 words)</i> Breast cancer metastasis to bone results in the loss of significant amounts of bone tissue through increased osteoclast activity. The purpose of this research is to determine what growth factors are secreted by tumors and how they influence osteoclasts. We have determined the tumors make IGF-I, IGF-II, M-CSF, PTHrP, TGF- β , and TNF- α . We are examining the effects of these growth factors on bone resorption and survival of avian osteoclasts and mouse osteoclast-like cells <i>in vitro</i> . We observe a similar stimulation of resorption activity by these factors between the two cell types. We have also observed a decrease in cell death with breast cancer cell conditioned media treatment which appears to be the result of TGF- β and/or TNF- α treatment. There are differences in the effects of the growth factors on attachment of the cells as IGF-I, IGF-II, PTHrP, and TNF- α all stimulate increased attachment of the mouse cells while having no effect on avian cell attachment. We are currently exploring whether this is due to differences in the state of differentiation of the two cell populations. Taken as a whole, we have made significant progress in defining the roles of these growth factors in stimulating tumor-driven osteolysis.			
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FOREWORD

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In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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PI - Signature Date
Wm J. Cursley 9/22/98

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INTRODUCTION

As detailed in the following report, we have made excellent progress on this project. We have initiated our studies of human tumors using samples provided by the Mayo Clinic, as outlined in the proposal. In addition, we have contacted local hospitals in the Duluth area to obtain fresh tumor samples. These will allow us to expand our studies to explant cultures for secreted growth factors. Appended to the end of this progress report are Institutional Review Board approvals for these additions. These studies are on-going and have already yielded exciting data which we are including in this Progress Report.

A major concern of the reviewers was our use of an avian cell system to study the effects of growth factors on human cells. Recognizing the wisdom of these concerns, we have invested a considerable amount of time in establishing a mammalian model system in our laboratory. The IACUC approval for the addition of this species is appended. Once the well-characterized system was established, we compared the results which we were obtaining with the authentic avian cells with the mouse osteoclast-like cells. The results of these comparisons are presented in the Body of this Progress Report. There are some differences between the avian cell response and that of the mouse cells. We are currently investigating whether this is the result of further differentiation in the presence of the growth factors or a species difference in responsiveness. Using this mouse model, we have initiated studies of the effects of growth factors on survival and have obtained interesting results. Taken as a whole, we have made excellent progress with these studies.

BODY

Methods

I. Analysis of Tumor Samples

A. Sample Recovery: Tumors were removed from patients undergoing reparative orthopedic surgery at the Mayo Clinic. Tissue was aseptically removed and placed in sterile Saline for transport on ice to the laboratory. Samples were frozen in dry ice and shipped to the PI's laboratory at University of Minnesota, Duluth.

B. Reverse transcriptase polymerase chain reaction: Samples were ground in liquid nitrogen and RNA was isolated as previously outlined (Pederson et al.). Reverse transcriptase polymerase chain reaction (rtPCR, Pederson et al.) analysis was carried out with the following primers:

Strand	Primer name	sequence	reference
sense	hIGFI(917)	GCCTCTCAAGAGGCCAC	Steenbergh
antisense	hIGFI(1396)	GGTTGCACATTAACTCATC	Steenbergh
sense	hIGFII(1)	GCTGACCTCATTTCCC	Gray
antisense	hIGFII(421)	AAGTAGAAGCCGCGGT	Gray
sense	hGMCSF(133)	CCTGAGTAGAGACACTGC	Miyatake
antisense	hGMCSF(423)	ACTCCTGGACTGGCTC	Miyatake
sense	hPTHrP(8)	CAGGGCAGCTTGGAAAG	Mangin
antisense	hPTHrP(611)	CTTCTTGCGAACCAAGG	Mangin
sense	hTNF α (372)	CCAGGGACCTCTCTCT	Nedwin
antisense	hTNF α (1210)	GGGCCTCCAGAACTCA	Nedwin
sense	hcathepsinK(281)	AAGTACCCCTGTCTCAT	Rantakokk
antisense	hcathepsinK(846)	TCCCAC TGCCAAAACC	Rantakokk

C. Titering of antibodies: We are currently using dot blots with Chemoluminescence to titer the antibodies for immunolocalization studies. This is being done by suspending MDA MB 231 cell pellets in sample lysis buffer and spotting onto Nitrocellulose. Antibodies are from R&D and the analysis kit is from Pierce (used according to Vendor directions).

II Animal model: Much of our previous work on osteoclast differentiation and activity has employed authentic avian osteoclasts. Recognizing that there are issues of relating avian studies to mammalian biology, we have developed the model of mouse osteoclast-like cell (mOCL) generation in our laboratory to extend our studies of mammalian osteoclast-like cells.

A. Change of Model: We are using a co-culture of mouse marrow and support cells as we have previously published to obtain mammalian osteoclast-like cells (mOCLs) (Clohisy et al.). Cells are generated by a modification of the method of Udagawa et al. using ST2 cells (RIKEN Cell Bank, Tsukuba, Japan) for support cells. ST 2 cells are plated at a density of 8×10^4 per well in 48 well plates. Following attachment, 5×10^2 marrow cells from Balb/C mice are added in Minimum Essential Media (MEM, Gibco) freshly supplemented with 50 $\mu\text{g}/\text{ml}$ ascorbic acid (Sigma Chemical Co.), 10% fetal bovine serum (Hyclone), non-essential amino acids (Sigma Chemical Co.), 10^{-7} M glucocorticoid (Sigma Chemical Co.), 10^{-8} M Vitamin D (Biomol), 100 U/ml penicillin (Sigma Chemical Co.), and 100 mg/ml streptomycin (Sigma Chemical Co.). Cultures are maintained in 5% CO₂ at 37° C for the length of time dictated by the specific experiments (usually 9-12 days)..

B. Viability: We have examined mOCL cultures for changes in the number of multinucleated tartrate resistant acid phosphatase (TRAP) positive cells with time. Following plating, the cells are fixed at various times with 1% paraformaldehyde in phosphate buffered saline and stained for TRAP activity (Sigma Leucocyte Acid Phosphatase kit). The number of TRAP positive cells is scored as reported previously (Clohisy et al.).

C. DNA fragmentation/apoptosis: We purify the TRAP positive cells as follows: We have discovered that, in our hands, contaminating cells are removed by treatment with 1 mg/ml each collagenase and Dispase in PBS for 5 minutes at 37°C. The released cells are removed and the plates washed with PBS. The remaining cells are removed with 5 mM EDTA in PBS. Cells are collected, centrifuged, resuspended in αMEM medium, and used for further analysis. The cultures are 90% pure multinucleated TRAP-positive cells. DNA fragmentation was detected using the TdT-FragEL detection kit (CALBIOCHEM) according to the manufacturers directions.

D. Attachment: Isolated avian osteoclasts and mOCLs were plated on bone slices with various treatment for 24 hours. Cultures were fixed, stained for TRAP activity, and the number of TRAP positive multinucleated cells scored as above.

Results

In this progress report, we address the specifics of each of the tasks which we outlined in the grant proposal and outline our progress in completing each of these aims.

Task 1: PCR analysis of tumors: Months 1-12

We have made excellent progress on this task. To determine what growth factors are being produced at the sites of osteolysis in patients, we examined metastatic breast tumors removed from bones. We have performed PCR analysis of tumors obtained from the Mayo Cancer Center as discovered that tumors express mRNA for IGF-I, IGF-II, M-CSF, PTHrP, TGF-β, and TNF-α. We have also initiated collaborations with physician organizations within the Duluth community to obtain samples (see appended copies of Internal Review Board approvals). This will allow us access to additional samples for PCR analysis as well as for Tasks 2 and 3.

Task 2: immunolocalization of factors in tumors: Months 5-16

We are currently developing the battery of antibodies necessary for these studies and determining their titers. To this end, we have performed dot blots on tumor cell extracts and have detected immunoreactive IGF-I, IGF-II, M-CSF, PTHrP, TGF-β, and TNF-α proteins in tumor samples from patients.

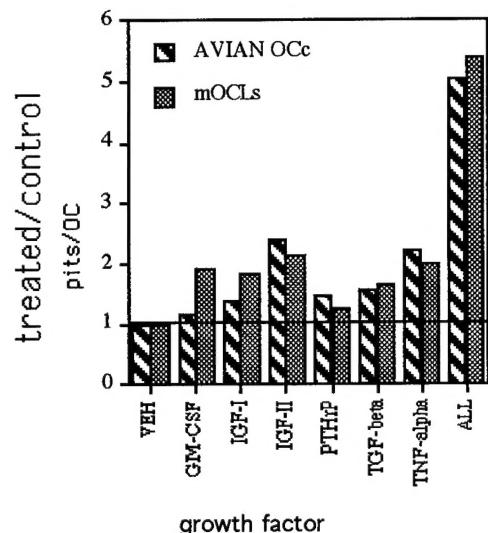
Task 3: examine tumors for the presence of factors: Months 17-36

Although this task was not scheduled to begin yet, we have initiated studies relating to it by examining the conditioned media of tumor explants for the presence of secreted proteins and have evidence that they secrete IGF-I, IGF-II, M-CSF, PTHrP, TGF-β, and TNF-α.

Task 4: examine the influence of GM-CSF, IGF-II, TNF-α, and PTHrP on viability: Months 1-8

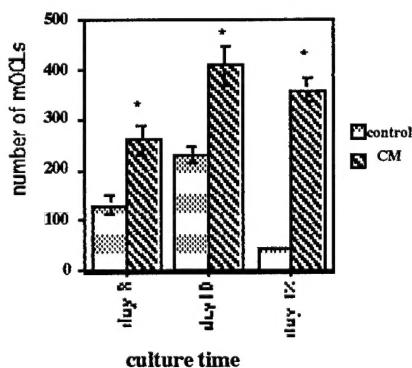
We have made excellent progress on this aim as follows: The reviewers expressed strong concerns about the choice of using avian model system for studying human diseases. We have therefore put extensive efforts into developing a mouse model. This model system is up and running and we have focused on obtaining relatively pure populations of cells which we are using for these studies. Our initial determinations of their use for these studies were focused on comparing the responses of these cells to the authentic osteoclasts which were the basis of this proposal. As demonstrated in Figure 1, murine osteoclast-like cells respond to growth factors in a manner similar to authentic avian osteoclasts.

Figure 1. Comparison of the effects of growth factors on resorption activity of avian osteoclasts and mOCLs was examined. Cultures were treated with the following concentrations of each factor: GM-CSF: 0.15 ng/ml; IGF-I: 100 ng/ml; IGF-II: 100 ng/ml; PTHrP: 10 nm; TGF- β 2: 0.2 ng/ml; TNF- α : 0.5 ng/ml. Cultures were fixed, stained for TRAP activity, and the number of TRAP positive multinucleated cells scored (1).



We have examined the effects of conditioned media from the 231 human breast cancer cell line on mOCL survival. To examine this, we maintained cells in the presence or absence of 231 conditioned media (Figure 2). In the absence of conditioned media, there was a peak in the number of mOCLs which declined with further culture. Examination of the influence of conditioned media on the survival of osteoclast-like cells suggested that the addition of the conditioned media resulted in no similar decrease in multinucleated TRAP-positive cells in the culture. These data support that breast cancer tumor cells may produce substances which are capable of promoting survival of osteoclasts.

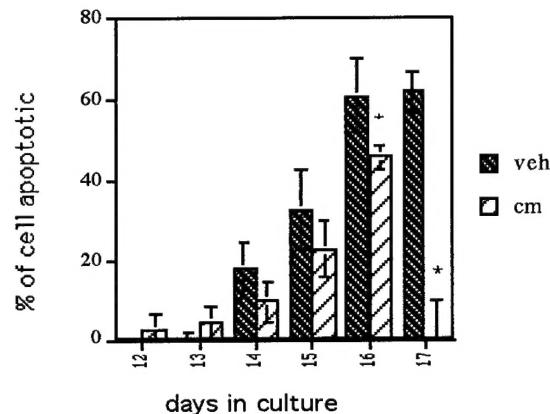
Figure 2. Effects of Breast Cancer Cell Conditioned Media on mOCL Survival. Mouse marrow was plated with ST2 cells and cultured in the presence or absence of 1% 231 cell conditioned media for the indicated time. Cultures were fixed, stained for TRAP activity, and the number of TRAP positive multinucleated cells scored (1). $P>0.01$.



Task 5: Examine the influence of GM-CSF, IGF-II, TNF- α , and PTHrP on apoptosis with a DNA fragmentation assay. Months 9-28

Having evidence that breast tumor cell conditioned media might be active in promoting viability of mOCLs, we next examined the effects of conditioned media on DNA fragmentation of the cultures (Figure 3). As demonstrated below, the levels of DNA fragmentation were consistently lower in the presence of 231 conditioned media on days 14 through 17 of culture.

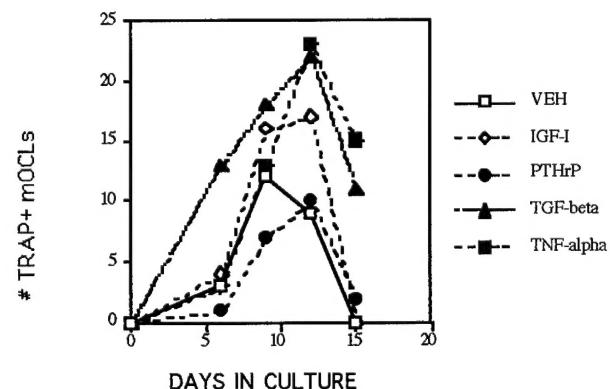
Figure 3. Detection of apoptotic nuclei in mOCL cultures. Each day beginning with day 12, cells were harvested with pronase/EDTA solution and replated on coverslips for 4 hours prior to fixation and analysis. P>0.05.



Our next goal is to examine the effects of GM-CSF, IGF-II, TNF- α , and PTHrP on apoptosis with this assay. These studies are ongoing.

Effects of growth factors on mOCL survival: We have initiated studies to examine the effects of IGF-I, PTHrP, TGF- β , and TNF- α on the survival of mOCLs in culture. Mouse marrow and ST2 support cells were plated as above and were treated beginning on day 0 with either growth factor or vehicle. Cultures were feed and treated every 3 days. On days 6, 9, 12, and 15 separate plates were harvested, fixed, and stained for TRAP activity (Figure 4). By day 6, there were more cells in the IGF-I, TGF- β , and TNF- α -treated cultures than in the control vehicle-treated cultures and even fewer in the PTHrP-treated cultures. Interestingly, by day 15 of culture, there were no mOCLs present in the vehicle, PTHrP, or IGF-I-treated cultures whereas the TGF- β and TNF- α -treated cultures maintained high levels of mOCLs. These data support that TGF- β and TNF- α may be promoting survival of mOCLs.

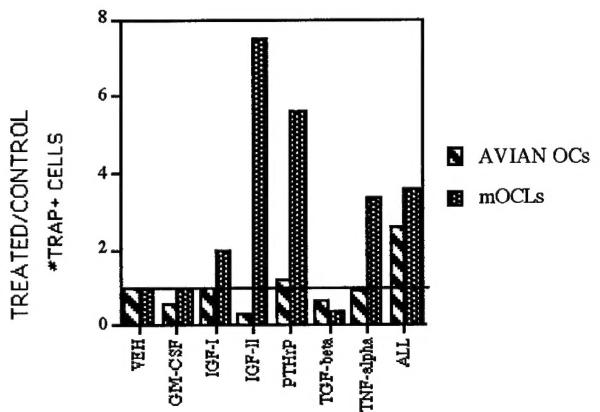
Figure 4. The effects of growth factors on differentiation and survival of mOCLs were examined. Cultures were set up as outlined above and treated with the following concentrations of each factor: IGF-II: 100 ng/ml; PTHrP: 10 nm; TGF- β : 0.2 ng/ml; TNF- α : 0.5 ng/ml. Cultures were fixed, stained for TRAP activity, and the number of TRAP positive multinucleated cells scored (1).



Task 6: examine the influence of GM-CSF, IGF-II TNF- α , and PTHrP on attachment. Months 1-12.

We have made excellent progress on this aim. As demonstrated in Figure 5, we have compared the effects of these factors on attachment of both avian osteoclasts and mOCLs. Interestingly, there was a much more pronounced effect of IGF-I, IGF-II, PTHrP, and TNF-alpha on mOCL binding than on binding of the avian osteoclasts. This may reflect an effect of the growth factors on differentiation stage of the cultures. We are currently investigating this hypothesis.

Figure 5. The effects of growth factors on binding of avian osteoclasts and mOCLs. Cultures were set up as outlined above and treated with the following concentrations of each factor: IGF-I: 100 ng/ml; IGF-II: 100 ng/ml; PTHrP: 10 nm; TGF- β 2: 0.2 ng/ml; TNF- α : 0.5 ng/ml.



Task 7: examine the influence of GM-CSF, IGF-II TNF- α , and PTHrP on integrin expression. Months 5-28.

Prior to examining integrin expression in the mammalian model system, it was important to determine if the growth factors under study influenced attachment of the mouse cells. The additional time spent establishing the mammalian system and doing the comparison of the attachment patterns of the mouse cells with the avian cells has precluded our examination of the effects of the growth factors on integrin expression. These studies are currently being designed and we expect to complete these studies within the time-frame allocated for them.

Task 8: examine the influence of GM-CSF, IGF-II TNF- α , and PTHrP on lysosomal enzyme secretion: Months 17-36.

These studies have not yet begun. Since we are just completing month 13 of this grant, they are not slated to begin yet.

CONCLUSIONS

The above studies demonstrate that we are making significant progress in our goal of identifying which factors that are produced by metastatic tumors stimulate osteoclastic activity. These data support that the tumors make a number of osteolytic growth factors including GM-CSF, IGF-I, IGF-II, PTHrP, TGF- β , and TNF- α . Our data suggest that all of these stimulate the mature cells to resorb bone. The data support that tumor cells conditioned media maintains survival of osteoclast-like cells and that this may be the result of the production of TGF- β and TNF- α . The possibility is also raised by our data that IGF-I, IGF-II, PTHrP, and TNF- α may be involved in promoting binding and/or further

differentiation of osteoclasts. Future experiments will focus on defining the mechanisms by which these growth factors influence osteolysis as detailed in our proposal.

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UNIVERSITY OF MINNESOTA

Twin Cities Campus

Research Subjects' Protection Programs

*Institutional Review Board: Human Subjects Committee (IRB)
Institutional Animal Care and Use Committee (IACUC)*

September 12, 1997

Box 820

*D528 Mayo Memorial Building
420 Delaware Street S.E.
Minneapolis, MN 55455-0392*

612-626-5654

Fax: 612-626-6061

E-mail: irbiacuc@ortta.umn.edu

Merry Jo Oursler
211 Life Sciences
University of Minnesota
Duluth, MN 55812

RE: "Breast Cancer Stimulation of Osteolysis"
Animal Subjects Code Number: 9611A00010

Dear Dr. Oursler:

The change in protocol for the referenced study that you submitted on August 25, 1997, was reviewed and approved. The study is now approved for the use of 644 Class A mice and 1170 Class A chickens.

As Principal Investigator of this study, you are required by federal regulations to inform the IACUC of any proposed changes to your research that involves animals. Changes should be reviewed and approved by the IACUC before they are initiated.

Thank you for keeping the IACUC informed of the status of your research.

If you have any questions, call the IACUC office at (612) 626-5654.

Sincerely,



Marie Holm
Assistant Director

MH

St. Luke's

Hospital & Regional Trauma Center

915 East First Street
Duluth, MN 55805
218/726-5555

20 May 1998

Merry Jo Oursler, Ph.D.
211 Life Sciences
University of Minnesota, Duluth
10 University Drive
Duluth, MN 55812

Re: "Identification of Growth Factors Present in Metastatic Breast Cancer Tumors – Breast Cancer Stimulation of Osteolysis"

Dear Dr. Oursler:

Thank you for the clarification of what is to be studied in the breast tissue being removed for clinical reasons. You may proceed with the above study.

Please inform the Committee of any adverse reactions occurring during the study. You are reminded that we will request an annual report of activities. Good luck with this research.

Sincerely,



James L. Anderson, M.D.
Chairperson, Human Studies Committee



Duluth Clinic

St. Mary's/Duluth Clinic
Health System

400 East Third Street
Duluth, Minnesota 55805
(218) 722-8364

May 13, 1998

Merry Jo Oursler, Ph.D.
Department of Biology
University of Minnesota-Duluth
211 Life Science Building
10 University Drive
Duluth, Minnesota 55812

RE: Breast Cancer Stimulation of Osteolysis
IRB #: 05-98-01

Dear Doctor Oursler:

Thank you for submitting your project entitled "Breast Cancer Stimulation of Osteolysis" to the SMDC Institutional Review Board office for review. As Chair of SMDC Institutional Review Board, I have administratively reviewed the application that was submitted to the IRB Human Subjects Committee at the University of Minnesota, the approval letters from the IRBs at the University of Minnesota and Mayo Clinic, and Optional Form 310 documents submitted by the University of Minnesota and Mayo Clinic. It has been determined that your study qualifies for exempt status and does not need to be formally presented to the SMDC Institutional Review Board for full review. Therefore, your research study will be registered with the SMDC Institutional Review Board and Research Office.

Best wishes for a successful study. Please forward the results of your research study to the SMDC Institutional Review Board upon completion of the study so the file may be closed.

Sincerely,

Clyde R. Olson, M.D.
Chair, SMDC Institutional Review Board

CRO/bj